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Platelet-derived Growth Factor-BB and Transforming Growth Factor Beta1 Selectively Modulate Glycosaminoglycans, Collagen, and Myofibroblasts in Excisional Wounds

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Recombinant platelet-derived growth factor (PDGF) and transforming growth factor beta1 (TGF-β1) influence the rate of extracellular matrix formed in treated incisional wounds. Because incisional healing processes are difficult to quantify, a full-thickness excisional wound model in the rabbit ear was developed to permit detailed analyses of growth-factor–mediated tissue repair. In the present studies, quantitative and qualitative differences in acute inflammatory cell influx, glycosaminoglycan (GAG) deposition, collagen formation, and myofibroblast generation in PDGF-BB (BB homodimer)- and TGF-β1-treated wounds were detected when analyzed histochemically and ultrastructurally. Although both growth factors significantly augmented extracellular matrix formation and healing in 10-day wounds compared with controls ($P < 0.002$), PDGF-BB markedly increased macrophage influx and GAG deposition, whereas TGF-β1 selectively induced significantly more mature collagen bundles at the leading edge of new granulation tissue ($P = 0.007$). Transforming growth factor-β1–treated wound fibroblasts demonstrated active collagen fibrillogenesis and accretion of subfibrils at the ultrastructural level. Myofibroblasts, phenotypically modified fibroblasts considered responsible for wound contraction, were observed in control, but were absent in early growth-factor–treated granulating wounds. These results provide important insights into the mechanisms of soft tissue repair and indicate that 1) PDGF-BB induces an inflammatory response and provisional matrix synthesis within wounds that is qualitatively similar but quantitatively increased compared with normal wounds; 2) TGF-β1 preferentially triggers synthesis and more rapid maturation of collagen within early wounds; and 3) both growth factors inhibit the differentiation of fibroblasts into myofibroblasts, perhaps because wound contraction is not required, due to increased extracellular matrix synthesis. (Am J Pathol 1991, 138:629–646)

Polypeptide growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor beta1 (TGF-β1) are potent inducers of normal soft tissue repair mechanisms and can reverse deficient repair rates.[1–4] These growth factors are released by platelets, monocytes/macrophages, endothelial cells and fibroblasts, cell types that are normally recruited into the wound and are required for normal wound healing.[2,3,5–8]

Normal wound healing has classically been considered to proceed in three phases: acute inflammatory, extracellular matrix and collagen synthesis, and collagen remodeling.[9–11] We have recently demonstrated that a single application of the BB homodimer of PDGF (PDGF-BB) to incisional wounds augmented the influx of neutrophils, monocytes, and fibroblasts in a time-dependent ordered sequence from days 1 to 21 after wounding.[2,8] In

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contrast, TGF-β1 treatment transiently increased wound macrophage and fibroblast influx only during the first week after wounding.[1,8] Because both growth factors increased the force required to disrupt the incisional wounds, it appeared that they influenced the final common pathway (ie, collagen synthesis), albeit by different mechanisms. New tissue formation and wound cellularity are difficult to quantify in the incisional model, however, because of the proximity of the wound to normal tissue.

To analyze the mechanisms of growth-factor–mediated tissue repair, a full-thickness open excisional dermal wound model in the rabbit ear was developed.[12] Unlike incisional repair in which opposing wound edges are reapproximated, excisional wound healing occurs by the synthesis of a new extracellular matrix (ie, granulation tissue) that fills the open defect; therefore, quantitative measures of cellularity and new tissue growth are feasible in this model. In preliminary studies, PDGF-BB or TGF-β1 treatments were found more effective in promoting wound closure, compared with control wounds or wounds treated with either epidermal growth factor or basic fibroblast growth factor.[12]

Excisional open wounds may also heal by contraction of granulation tissue, a poorly understood process more important in loose-skinned animals, but also a contributor to repair in man.[11] Contractile forces leading to closure are considered to be mediated by myofibroblasts, smooth musclelike cells thought to be differentiated from wound fibroblasts.[13–20] The influence of growth factors on the generation of myofibroblasts and extracellular matrix constituents in open excisional wounds has not been studied at the light microscopic or ultrastructural levels. In the present studies, PDGF-BB treatment induced significantly more wound macrophages and PDGF-BB and TGF-β1 both induced significantly fewer myofibroblasts, but more wound fibroblasts. Transforming growth factor β1 appeared to induce a more rapid maturation of wound collagen, whereas PDGF-BB markedly enhanced the deposition of glycosaminoglycans normally observed in control wounds. Thus these studies demonstrate significant differences in the acute inflammatory and matrix deposition phases of wound healing in wounds treated with known potent vulnerary agents.

Methods

Rabbit Ear Ulcer Model

Six-millimeter wounds were made through the perichondrial membrane to the bare cartilage in New Zealand white (NZW) rabbits (Irish Farms, Norco, CA), as previously described.[12] Briefly, rabbits were anesthetized with 60 mg/kg ketamine (Parke Davis, Morris Plains, NJ) and 10 mg/kg xylazine (Haver-Mobay Corp., Shawnee, KA), prepared, and ears secured on plexiglass immobilizer in a sterile field. The anterior dermis was hydrodissected from the cartilage using 2% lidocaine containing epinephrine (1:100,000) (Astra, Westborough, MA). Four full-thickness dermal wounds were placed on each ear using a calibrated 6-mm trephine (Roboz Surgical Instrument Co., Washington, DC), leaving intact cartilage. Growth factors or buffer alone were applied to each wound, and each wound was immediately covered with an individual piece of occlusive dressing (Tegaderm, 3M, Minneapolis, MN). Growth-factor–treated and control wounds were placed at least 1 cm apart on the same ear; no leakage of growth factors into control wounds was detected.

In a preliminary experiment, the degree of wound contraction was assessed on 10 wounds using India ink reference points and a calibrated micrometer. Wounds were measured in triplicate by two investigators on multiple days after wounding. A maximum of 3% contraction occurred from days 10 to 20 after wounding; thus this is a minimally contracting excisional wound model.

Growth Factors

Recombinant PDGF-BB and TGF-β1 were purified to homogeneity as described[8] and were endotoxin free. Growth factors were delivered a single time to wounds in a 5-μl volume in sodium acetate buffer containing 0.25% albumin. The optimal doses were 5 μg for PDGF-BB and 1 μg for TGF-β1, based on preliminary dose-response curves (data not shown), and previous data in which growth factors were delivered to wounds in a collagen vehicle.[12] No differences in efficacy have been observed between PDGF-BB or TGF-β1 delivered in either aqueous vehicles or a collagen suspension (unpublished observations).

Histologic and Morphometric Analyses

Routine hematoxylin and eosin (H&E) staining was performed on cross-sections of all bisected wounds after 10 or 21 days for subsequent morphometric analyses as described.[12] One half of each wound was fixed in Omnifix (Xenetics Biomedical, Irvine, CA) and embedded in paraffin; half was processed for possible electron microscopic evaluation. A calibrated lens micrometer was used by two blinded, independent observers to measure the granulation tissue depth and gap of each bisected wound. From the granulation tissue gap and the original diameter of the wound, the new granulation tissue (NGT) influx, area, and volume were calculated. For each treatment group, 10 to 14 wounds from a total of five rabbits

were quantitatively evaluated on days 10 and 21 after wounding.

Alcian blue staining of sulfate groups in tissue sections was performed using routine methods at pH 2.5. Under these conditions, all glycosaminoglycans, including hyaluronic acid and highly sulfated proteoglycans, were identifiable. In a representative sample of five 10-day wounds per group, alcian blue staining was performed at pH 1 or pH 4, to more specifically identify highly sulfated proteoglycans or hyaluronic acid, respectively, within PDGF-BB, TGF-β1, and untreated wounds.[21]

New collagen synthesis was detected using polarization optics (Optiphot, Nikon, Inc., Garden City, NY) and Sirius red staining on 12-μ-thick sections.[22,23] Collagen bundles and fibers/fibrils were distinguished on the basis of their marked size differences and birefringent color under polarized light (bundles = red; fibers/fibrils = green to yellow to red) at 20× magnification, and were evaluated by two independent, blinded observers. The frequency of collagen bundle influx into the original wound margin, and the frequency of bundles or fibrils at the leading edge of new tissue, were calculated for each treatment group. The distance between collagen bundles at opposing wound edges was measured across the bisected wound using a lens micrometer, as described previously.

Statistical Analysis

For light microscopy morphometric analyses, 10 to 14 PDGF-BB, TGF-β1, or control wounds from five rabbits were analyzed by H&E, alcian blue, and Sirius red stains. The unpaired Student's *t*-test with a multiple comparisons correction was used to analyze differences in collagen bundle distances across the wound, and chi-square analysis of proportional data was employed to detect differences between the frequency of wounds containing collagen bundles or fibrils between treatment groups (Statview SE, Brainpower, Calabasas, CA).

Electron Microscopic Analysis of Wounds

Two representative bisected wounds per treatment group at days 10 and 21 were selected for detailed electron microscopic (EM) analysis. Three 2-mm core biopsies were obtained from the leading edge of each wound, placed in coded containers, fixed in 2% glutaraldehyde in 0.2 mol/l (molar) phosphate buffer (pH 7.4), washed, and placed in osmium tetroxide in 0.2 mol/l phosphate buffer (pH 7.4) as described.[16] The tissues were dehydrated through a graded series of ethanol and propylene oxide and embedded in EM Bed 812 (Electron Microscopy Sciences, Fort Washington, PA). Toluidine-blue–stained thick sections (1 μ) from each wound were examined for wound orientation and selection of representative samples of the wound from the epidermis to the underlying cartilage for ultrastructural analysis. Uranyl-acetate– and lead-citrate–stained thin sections (60 nm) were mounted on unsupported copper 200 mesh grids, examined, and photographed in a Zeiss EM-10B electron microscope.

Representative wounds for EM analysis were selected based on the morphometric measurements obtained under light microscopy on the other half of the bisected wound. The biopsies were obtained at the advancing edge of new granulation tissue, and not from more mature, healed areas of the wounds. At least four grids containing 10 to 15 thin sections were cut from each sample block (biopsy). To minimize sampling error, a series of low-magnification micrographs were made from the epidermis to the underlying cartilage of each wound near the advancing edge of new granulation tissue. High-magnification records of selected areas were also obtained from these tissues. Myofibroblasts were defined ultrastructurally,[14,15] and distinguished from fibroblasts by the presence of 6- to 8-nm microfilament bundles with electron-dense bodies oriented longitudinally in the cytoplasm subjacent to the cell membrane, as described.[15] All ultrastructural data were obtained by one electron microscopist (J. Vande Berg) blinded to the treatments the wounds received.

Results

Morphometric Analysis of Healing

By day 10 after wounding, a single application of either PDGF-BB or TGF-β1 at the time of surgery significantly increased the depth, area, and volume of new granulation tissue (Table 1). The predominant cells in the wounds at day 10 were fibroblasts, although significant numbers

Table 1. *Depth and Volume of New Granulation Tissue (NGT) Influx into PDGF-BB or TGF-β1–Treated Wounds at 10 Days*

Wound treatment*	NGT depth (μ)	NGT volume (mm^3)
PDGF-BB	821 ± 55† (150)	15.3 ± 2.0† (174)
TGF-β1	796 ± 46† (145)	15.1 ± 1.0‡ (172)
Control	549 ± 30	8.8 ± 0.7

* Mean ± SE, n = 10–14 per group; numbers in parentheses indicate the percentage of untreated (control) wound values. Unwounded dermis is 434 ± 31 μ thick, and has a volume of approximately 10.7 mm^3.

† $P < 0.0001$, unpaired one-tailed *t*-test, growth factor-treated versus control.

‡ $P < 0.002$.

of mononuclear cells were observed also in PDGF-BB–treated wounds (see below). The original volume of control wounds was nearly replaced by day 10, and was significantly increased in growth-factor–treated wounds (Table 1), indicative of augmented as well as accelerated extracellular matrix production at the advancing wound margins. More than 80% of all control or growth-factor–treated wounds were fully reepithelialized at day 10. By day 21, histologic analysis of bisected wounds disclosed a closed wound consisting of a bed of maturing granulation tissue covered by epithelium.

Analysis of Wound Glycosaminoglycans and Collagen by Light Microscopy

Preliminary experiments indicated that both PDGF-BB and TGF-β1 enhanced open wound closure.[12] To determine mechanisms of growth-factor–augmented repair, extracellular matrix was analyzed by light microscopy using specific histochemical stains.

Alcian blue histochemical staining of sulfate moieties indicated the nonpolarizing extracellular matrix at all advancing wound margins consisted largely of glycosaminoglycans, which were greatly increased in PDGF-BB–treated wounds, compared with TGF-β1–treated and control wounds (Figure 1). When performed at pH 1 or pH 4 to enhance detection of proteoglycans or hyaluronic acid, respectively, alcian blue staining showed the enhanced GAG deposition in PDGF-BB–treated wounds consisted largely of hyaluronic acid. These results were confirmed using hyaluronidase pretreatment of selected wound sections to abrogate hyaluronic-acid–specific staining.[21]

Collagen from a series of 10-day-old growth-factor–treated and control wounds was analyzed by light microscopy using polarization optics and the Sirius red stain to enhance collagen's natural birefringence (Figure 2). Morphometric analysis showed strikingly increased numbers of new collagen bundles in 10-day TGF-β1–treated wounds (Figure 3A; in 100% of TGF-β1–treated wounds; $P = 0.06$, TGF-β1 *versus* control). New collagen bundles were more frequently observed at the advancing edge of incoming granulation tissue in TGF-β1–treated wounds compared with controls, (63% *versus* 9%, $P = 0.008$; Figure 3A). These bundles were similar in size to the natural collagen bundles observed in unwounded dermis (ie, mature collagen; Figure 2). In contrast, PDGF-BB–treated wounds demonstrated significantly fewer collagen bundles within the new granulation tissue ($P = 0.02$), a larger distance between bundles advancing into the wounds from opposing margins ($P = 0.007$), and none at the leading front of incoming tissue (Figures 3A and 3B). PDGF-BB-treated and control wounds, which also did not contain collagen bundles at the advancing wound margin, had similar amounts of thin collagen fibrils extending to the advancing wound edge (Figure 2), although the volume of new matrix deposited in PDGF-BB–treated wounds was nearly twofold greater than that observed in controls (Table 1). Thus, the matrix in 10-day TGF-β1–treated wounds contained predominantly new collagen, which extended to the advancing wound edge, whereas PDGF-BB–treated wounds had considerably more nonpolarizing granulation tissue at the advancing wound edge.

Of importance, GAGs and collagen bundles occupied reciprocal areas of all wounds; GAGs were less prominent in the older, more mature wound granulation tissue (Figures 1 and 2). By 21 days, PDGF-BB–treated wounds contained less GAGs and more collagen bundles (unpublished observations), indicating a normal sequence of maturation of the augmented provisional matrix observed 10 days after wounding.

Ultrastructural Comparison of Collagen Synthesis at 10 Days

Ultrastructural analysis was employed to confirm and extend the histologic observation of increased collagen bundles at the leading edge of TGF-β1–treated wounds. Six core biopsies at the advancing edge of new granulation tissue in two representative wounds either untreated or treated with PDGF-BB or TGF-β1 were examined 10 days after wounding. To minimize potential sampling errors, the entire leading edge of new granulation tissue was initially scanned. Most of the fibroblasts in granulation tissue from all wounds displayed well-developed endoplasmic reticulum in which many of the cisternae contained filaments or electron-dense material in various stages of distension. Markedly increased numbers of actively synthesizing and secreting fibroblasts were detected in PDGF-BB– and TGF-β1–treated wounds, however, as observed using light microscopy (Figure 1).

Transforming growth factor-β1–treated wounds contained the greatest amount of cellular activity characteristic of collagen synthesis, confirming the data obtained using Sirius red (Figure 4A). Sections of some fibroblasts showed multiple regions of Golgi bodies from which secretory vesicles could be traced to the cell membrane. In these areas apparent microfibrils were prominent just inside the cell membrane and subjacent to the membrane in the extracellular matrix, indicating active fibrillogenesis. An increasing gradient of collagen fibril diameters was associated with increasing distances from the cell (Figure 4A). In cross-section, microfibrils appeared to

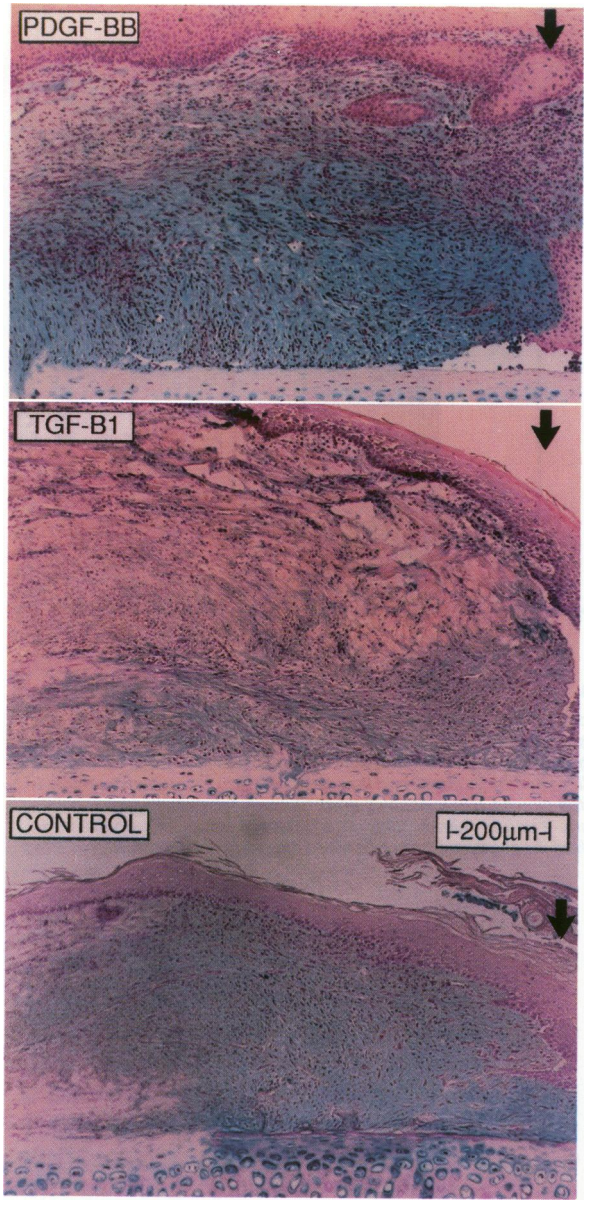

Figure 1. *Glycosaminoglycan content in PDGF-BB, or TGF-β1, or untreated control wounds. Alcian blue histochemical staining at pH 2.5 was used to detect GAG staining. The nonpolarizing matrix in all wounds contained GAGs, which were considerably more abundant in PDGF-BB–treated wounds. Arrows indicate the advancing edge of the wound. Wounds are the same as those presented in Figure 2. Note the inverse relationship between polarized collagen and GAG staining (Bar, 200 µ).*

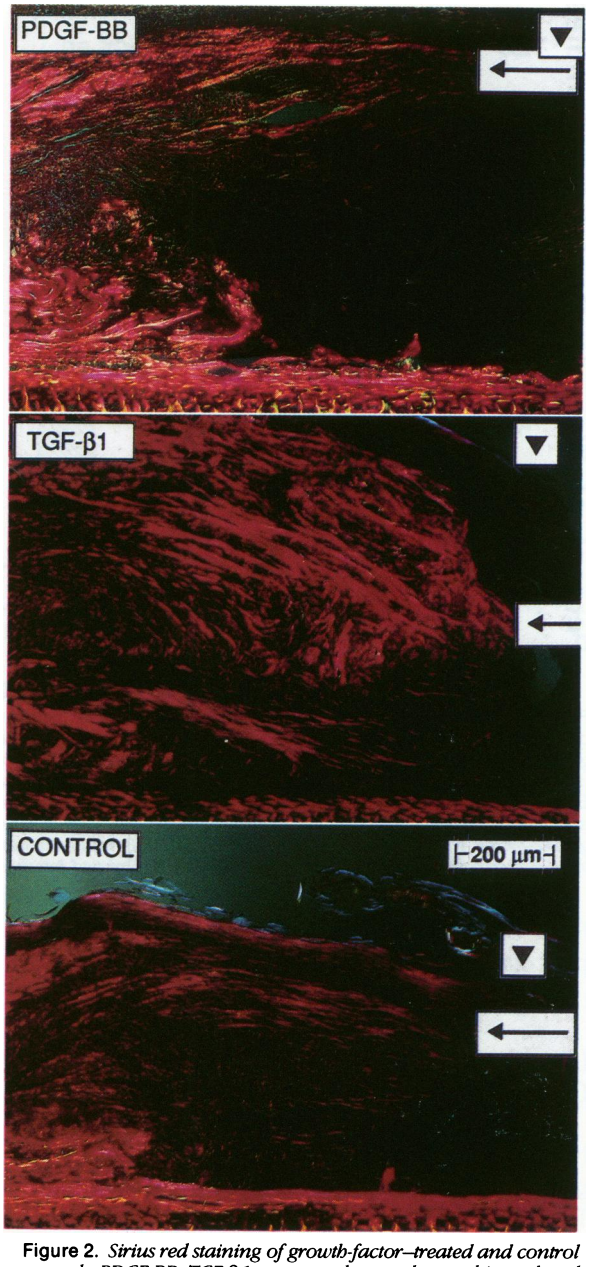

Figure 2. *Sirius red staining of growth-factor–treated and control wounds. PDGF-BB, TGF-β1, or control wounds were bisected and viewed under polarization optics for collagen bundles (red; 50 to 100 µ diameter, arrow) and collagen fibrils (yellow to green to red, arrows). Note the increased depth of new granulation tissue in growth-factor–treated wounds (see Table 1) and the inverse relationship between polarized collagen and GAG staining (see Figure 1). Arrowheads indicate epithelium at the advancing edge of the wounds (Bar, 200 µ).*

be adding to the diameters of collagen fibrils by accretion (Figure 4B). Other cell perimeters displayed fibril outgrowth from membranous indentations into the extracellular matrix (Figure 4C).

Platelet-derived growth factor-BB–treated and control wounds also demonstrated multiple regions of Golgi bodies within fibroblasts (Figure 5). As in the TGF-β1–treated tissues, secretory vesicles could be followed to the cell perimeters, where collagen microfibrils appeared to accumulate. Unlike TGF-β1–treated wounds, morphologic indications of direct collagen accretion in PDGF-BB–treated or control granulation tissue were not observed. While minimal ultrastructural differences between control and PDGF-BB–treated wounds were observed, the number of wound fibroblasts demonstrating active protein synthesis were considerably increased in the

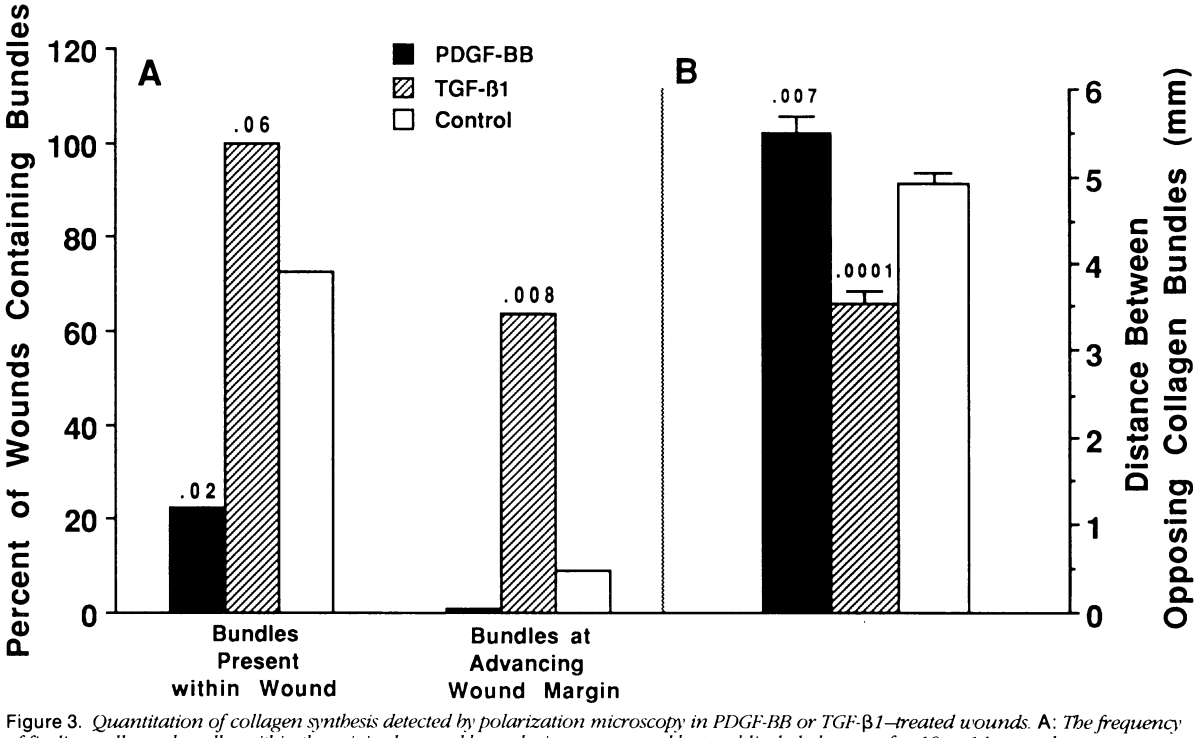

Figure 3. *Quantitation of collagen synthesis detected by polarization microscopy in PDGF-BB or TGF-β1–treated wounds.* A: *The frequency of finding collagen bundles within the original wound boundaries was assessed by two blinded observers for 10 to 14 wounds per treatment group.* P *values for differences between growth-factor–treated and control wounds are displayed above each bar and were obtained by chi-square analysis. All TGF-β1–treated wounds contained collagen bundles, and the majority had bundles at the advancing wound margin.* B: *The distance between collagen bundles on opposing sides of the bisected wound was measured quantitatively for growth-factor–treated and control wounds. The original wound diameter was 5.69 ± 0.04 mm.* P *values (above bars) were obtained using an unpaired t-test, growth-factor–treated versus control wounds. PDGF-BB–treated wounds had the greatest distance between opposing collagen bundles, while the distance between bundles was shortest in TGF-β1–treated wounds.*

PDGF-BB–treated wounds, as observed previously in explant cultures of wounds.[12]

Ultrastructural Analysis of 10-day Wounds: Cellular Content

Histologic analyses of PDGF-BB–treated wounds suggested an increase in inflammatory cells and fibroblasts. To confirm and extend these observations, ultrastructural analysis of healing wounds was performed. PDGF-BB–treated wounds were more cellular and contained a significantly increased percentage of inflammatory cells, compared with TGF-β1–treated or control wounds (Figure 6). PDGF-BB wound samples contained numerous monocytes and macrophages with scattered eosinophils, basophils, and lymphocytes, and interspersed random fibroblasts (Figure 6A). The ratio of inflammatory cells to fibroblasts within the entire depth of PDGF-BB–treated wounds was approximately 1:3 or 35% (Table 2). In TGF-β1–treated wounds, fibroblasts predominated, and overall less cellularity was detected than in wounds treated with PDGF-BB (Figure 6B). Approximately one of four cells (23%) were macrophages or other inflammatory cells, which was significantly increased compared with control wounds, but significantly decreased relative to PDGF-BB–treated wounds (Table 2).

In contrast to growth-factor–treated wounds, the granulation tissue of control wounds was less cellular and appeared more heterogeneous and disorganized; fibroblasts, inflammatory cells, and scattered fragments of collagen fibrils and fibrin deposits were detected (Figure 6C). For every 10 fibroblasts, approximately one inflammatory cell was observed; macrophages were infrequently found (Table 2). Control wounds showed markedly less cell proliferation, cell density, and extracellular matrix synthesis, compared with wounds treated with PDGF-BB or TGF-β1, consistent with previous observations made on cultured wound explants.[12]

Ultrastructural Analysis of Wounds at Day 21

Wounds were fully healed by 21 days after wounding; fibroblasts were the predominant cell type. Overall the density of fibroblasts and inflammatory cells was diminished relative to day 10. In PDGF-BB–treated wounds, some increased collagen synthesis was demonstrated, with the appearance of loosely packed collagen fibrils arranged in patterns of random mesh, or parallel in bun-

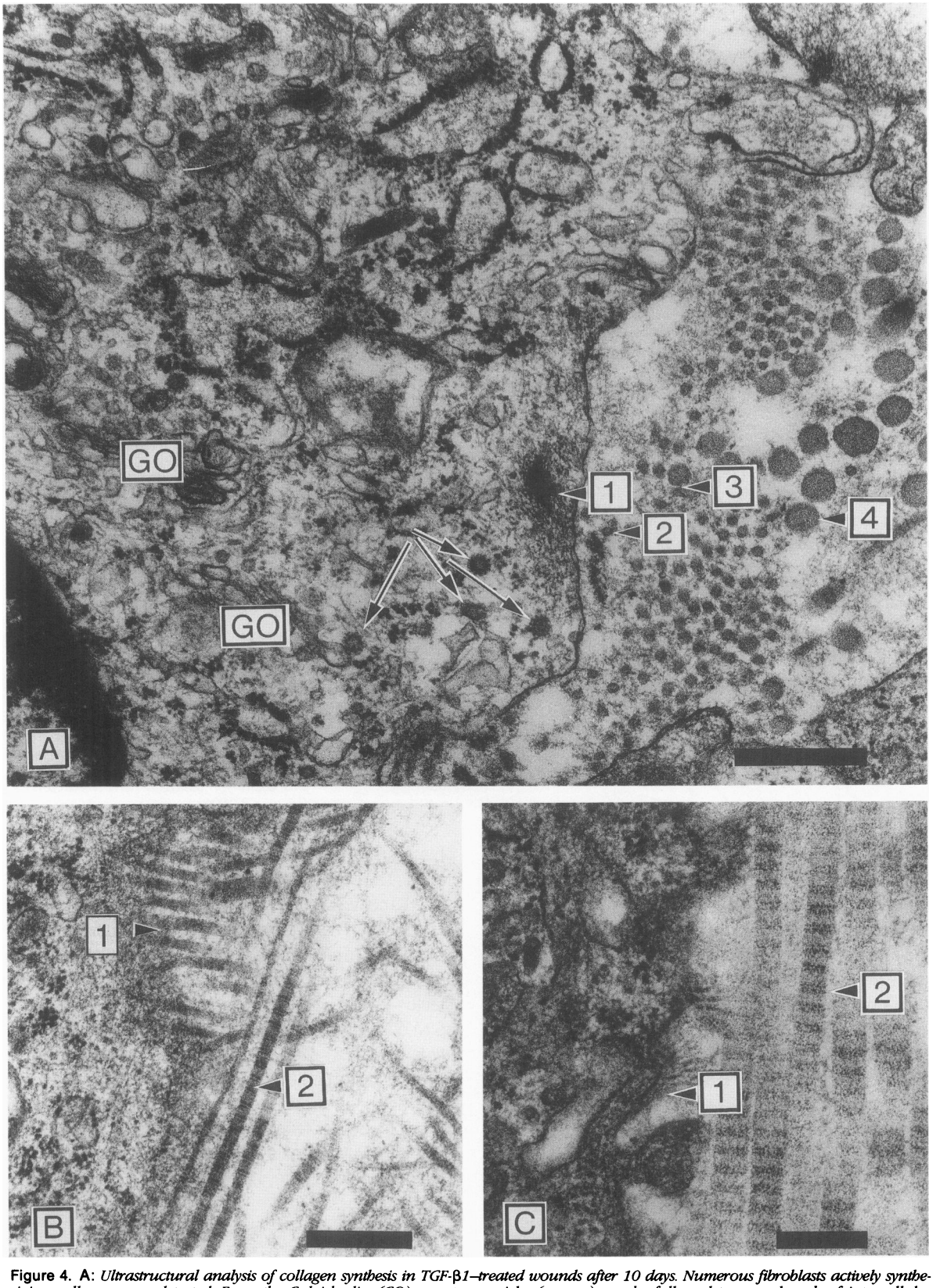

Figure 4. A: *Ultrastructural analysis of collagen synthesis in TGF-β1–treated wounds after 10 days. Numerous fibroblasts actively synthesizing collagen were detected. From the Golgi bodies (GO), secretory vesicles (arrows) can be followed to a meshwork of intracellular microfibrils (1) along the cell membrane. Adjacent to this area within the extracellular matrix, microfibrils (2) appear to increase in diameter with increasing distance from the cell (3 and 4) (Bar, 0.5 μ; ×44,000).* B: *Longitudinal section of a fibroblast-synthesizing collagen in a TGF-β1–treated wound. Collagen fibrils protruding from the cell membrane (1) exhibit an axial periodicity of 67 nm (2) (Bar, 0.5 μ; ×44,000).* C: *Microfibrils displaying evidence of periodicity (1) appear joined to mature collagen fibrils (2) in a wound treated with TGF-β1 (Bar, 0.25 μ; ×68,000).*

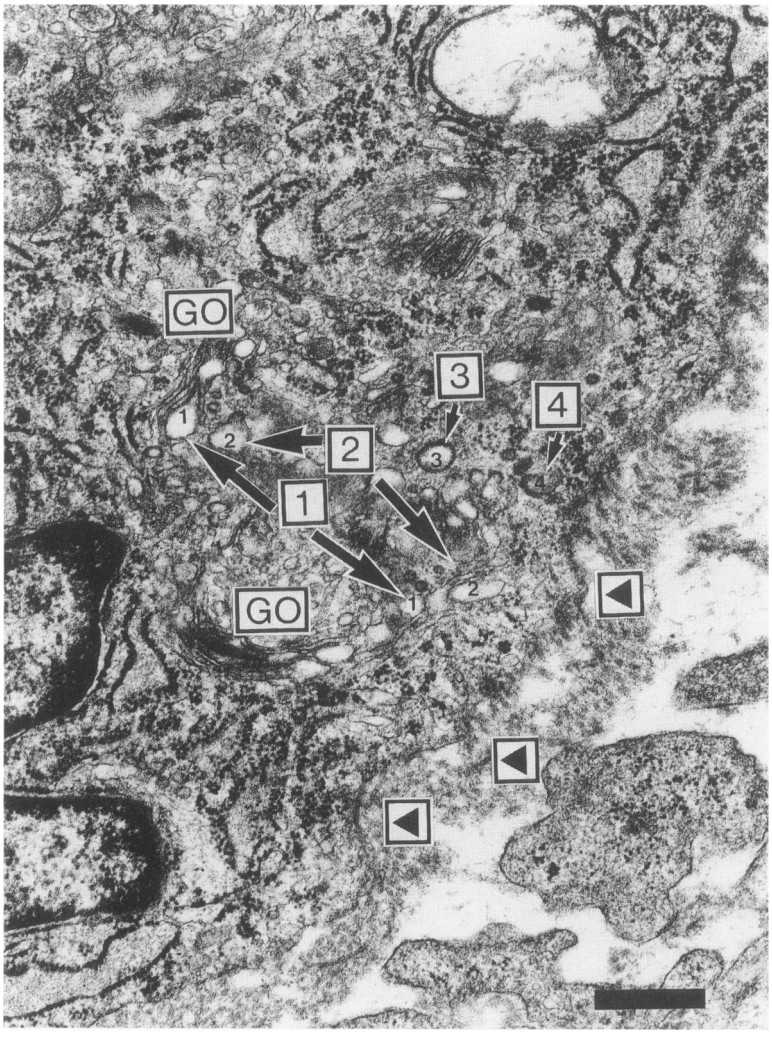

Figure 5. *Protein synthesis in fibroblasts from 10-day PDGF-BB–treated wounds. Fibroblasts exhibited prominent Golgi bodies (GO) from which secretory vesicles (1, 2, 3, and 4) can be followed to the cell membrane. In the extracellular matrix adjacent to the cell membrane, microfibrils begin to display an axial periodicity for collagen (arrowheads) (Bar, 0.5 μ, ×33,800).*

dle form (Figure 7A). Increased numbers of proliferating fibroblasts were detected, compared with TGF-β1–treated or control wounds. In TGF-β1–treated wounds, fibroblasts were arranged in parallel layers throughout the wound (Figure 7B). Considerably greater amounts of collagen were arranged in compact bundles of fibers in a developing orthogonal array. The extracellular matrix of control wounds contained increased numbers of collagen fibrils, but most of these structures were loosely packed and disorganized, and fewer were arranged in the bundles characteristic of TGF-β1–treated wounds (Figure 7C).

Electron Microscopic Detection of Myofibroblasts

Myofibroblasts were identified by their characteristic ultrastructural appearance in day 10 and day 21 wounds (see Methods). Notably none of the fibroblasts demonstrated evidence of phenotypic modulation to myofibroblasts in PDGF-BB– or TGF-β1–treated wounds at 10 days (Table 2). Approximately 12% of the fibroblasts in day 10 control wounds had modulated to a myofibroblast phenotype. The cytoplasm of these cells demonstrated prominent microfilament bundles characteristic of ultrastructurally defined myofibroblasts (Figure 8A). In growth-factor–treated wounds, a very small percentage (2.5% to 5%) of fibroblasts demonstrated a myofibroblast phenotype at day 21 (Table 2). The myofibroblasts in these wounds contained microfilament bundles that were considerably less developed and less prominent than those in myofibroblasts found in day 10 control wounds (Figure 8B). No myofibroblasts were observed in any of the control biopsies by 21 days (Table 2).

Discussion

Both PDGF-BB and TGF-β1 are potent vulnerary agents that initiate increased collagen synthesis over different periods in rat incisional wounds.[8] In the present studies,

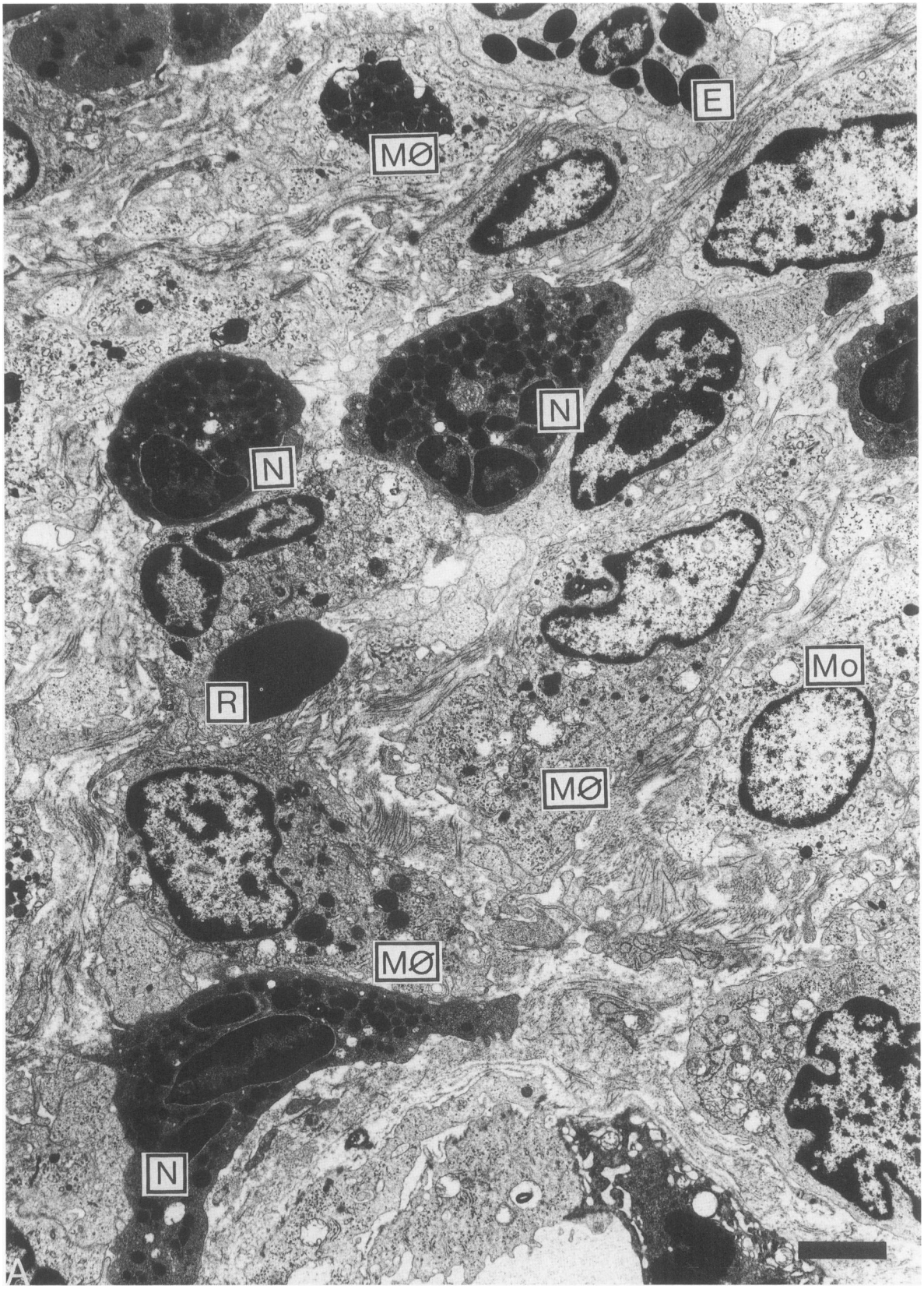

Figure 6. A: *Cellularity of a PDGF-BB–treated excisional wound after 10 days. Monocytes (Mo) and macrophages (MØ) were the predominant inflammatory cells represented in wounds; other cells included neutrophils (N) and eosinophils (E). Occasional lymphocytes and red blood cells (R) also were observed. Fibroblasts also were present but tended to predominate toward the wound base. Increased overall numbers of cells were present in PDGF-BB–treated wounds, compared with TGF-β1–treated and control wounds. The extracellular matrix contained loosely organized collagen fibrils (Bar, 3 μ; ×5500).* B: *(Page 638). Cellular content of TGF-β1–treated excisional wounds after 10 days. Fibroblasts (F) predominated within an extracellular matrix that was composed of collagen fibrils (CO) organizing into maturing collagen fibers. Inflammatory cells were less prominent but increased above control. This micrograph was chosen to demonstrate the increased collagen deposition (Bar, 3 μ; ×5500).* C: *(Page 639). Cellularity and matrix content of untreated (control) wounds after 10 days. Macrophages (MØ), monocytes (Mo) lymphocytes (L), neutrophils (N) and fibroblasts (F) were observed in an extracellular matrix that was considerably less developed and contained fewer cells than wounds treated with PDGF-BB and TGF-β1. Fewer inflammatory cells were observed than in growth-factor–treated wounds (Bar, 3 μ; ×5500).*

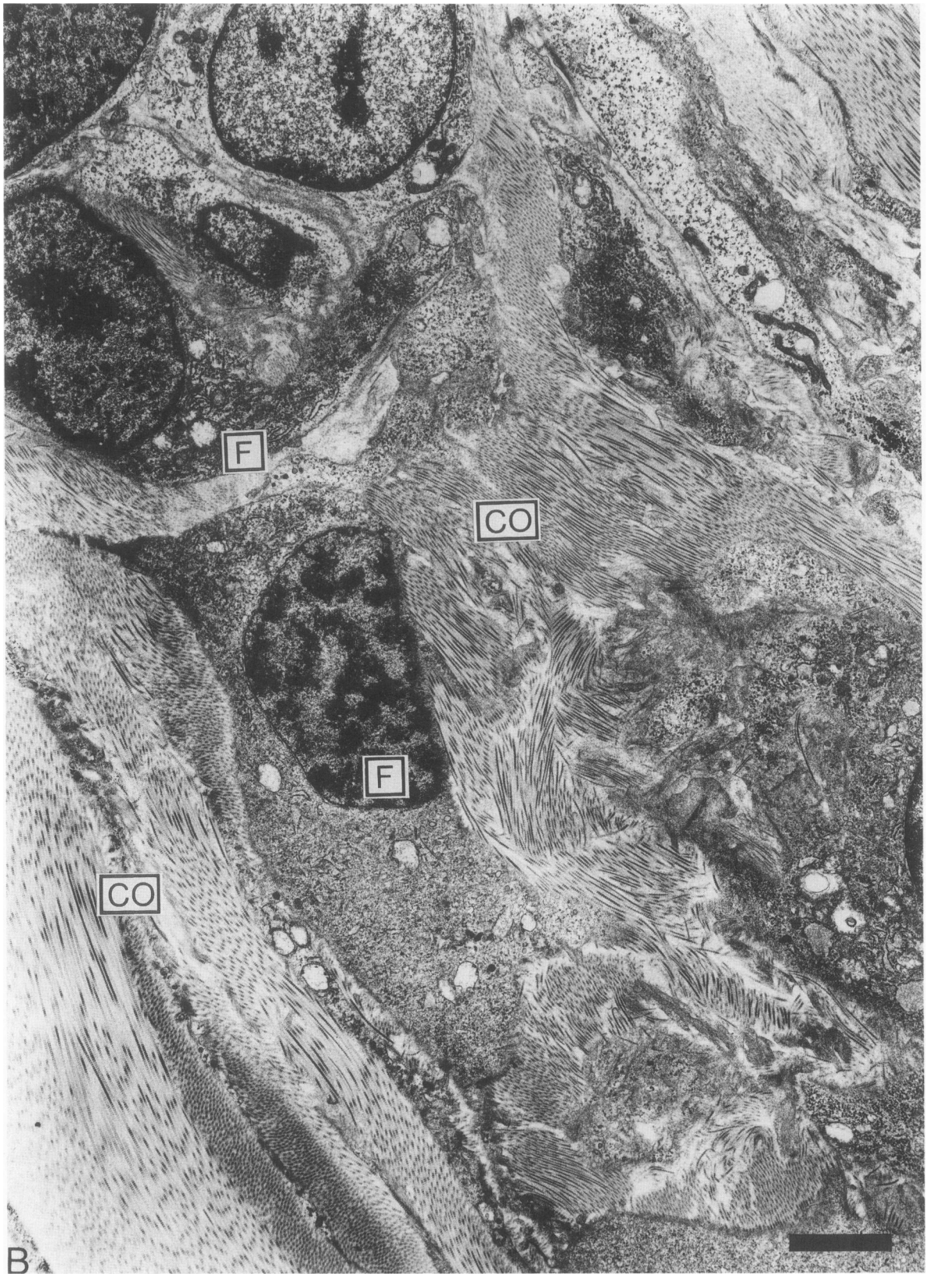

Figure 6B.

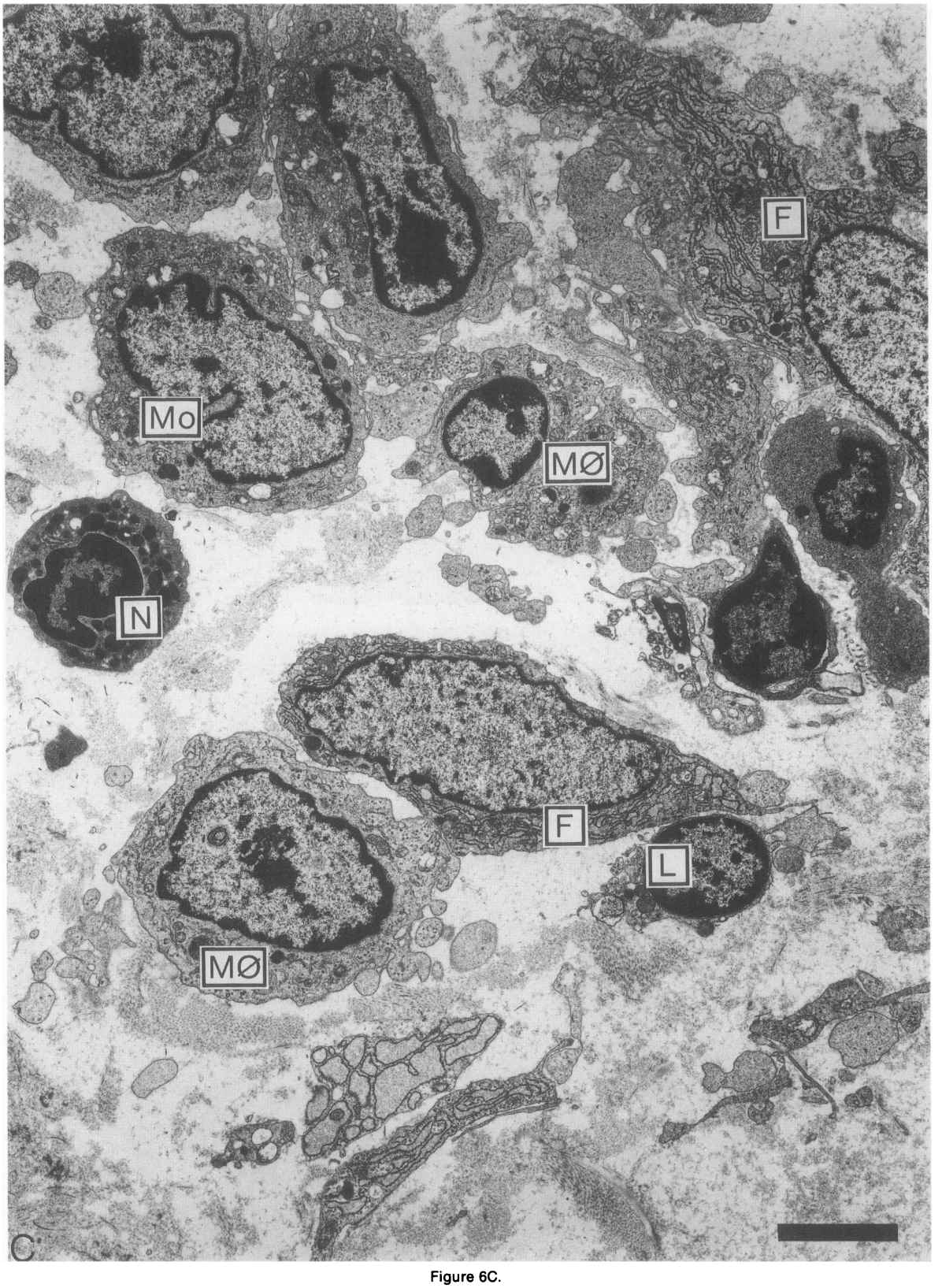

Figure 6C.

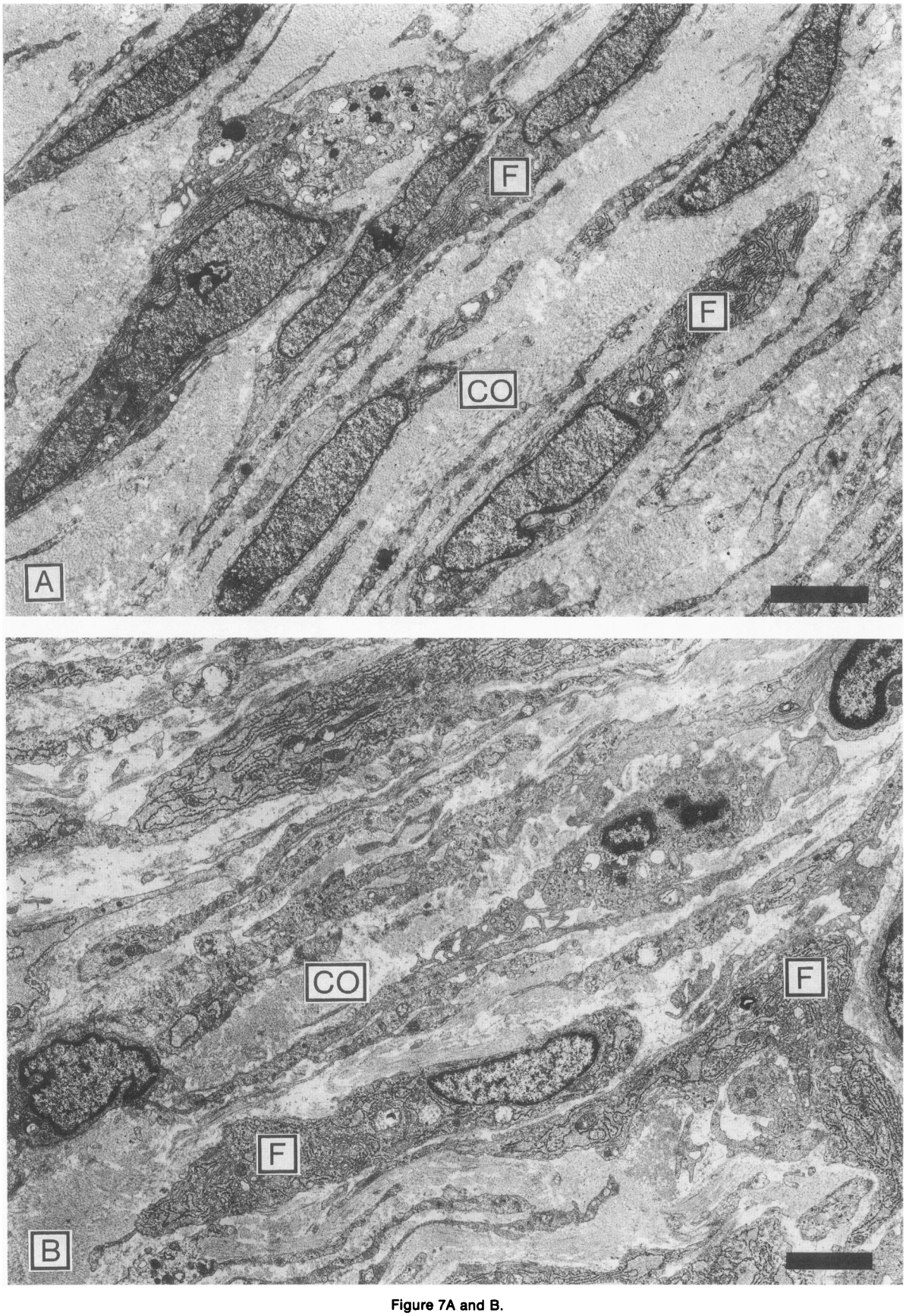

Figure 7A and B.

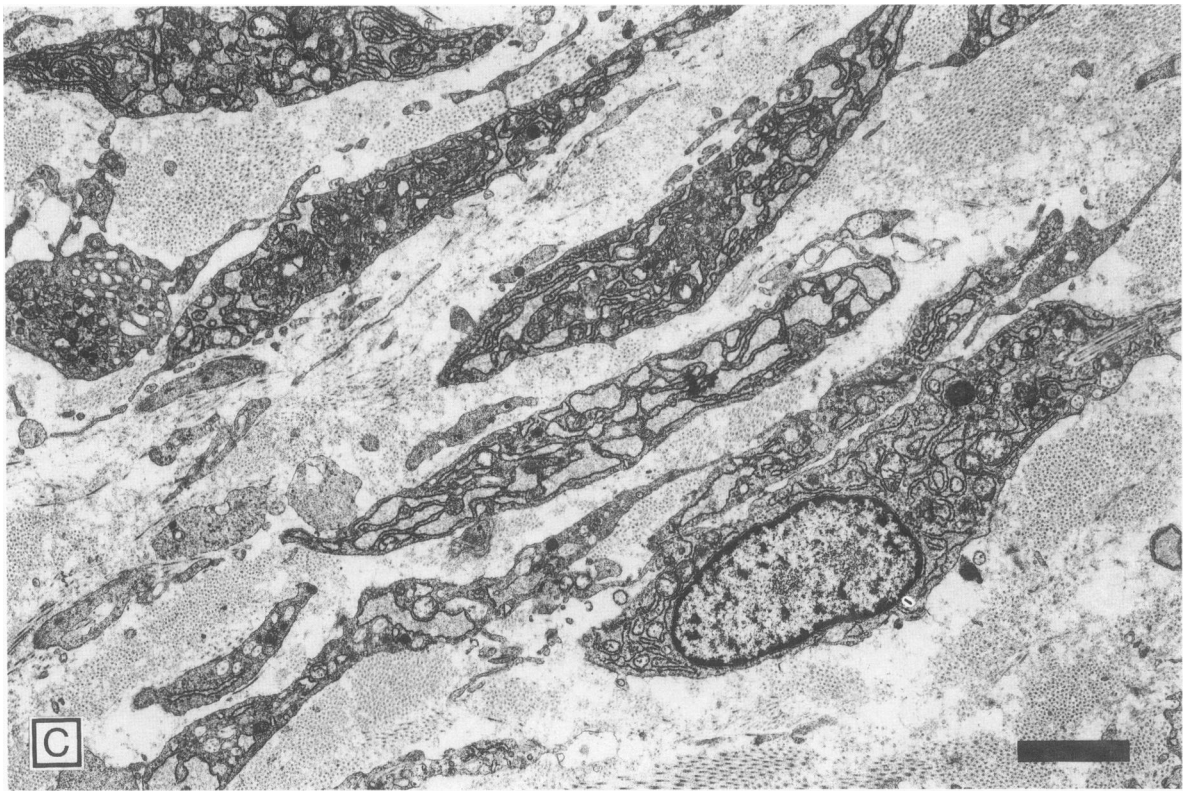

Figure 7. *Cellularity and matrix content in 21-day excisional wounds.* **A:** *(Page 640). PDGF-BB–treated wounds contained primarily fibroblasts in parallel arrays within a collagen-containing matrix that was less dense than in TGF-β1–treated wounds (Bar, 3 μ; ×5500).* **B:** *(Page 640). TGF-β1–treated wounds contained numerous fibroblasts (F) that were well organized into parallel arrays within a dense collagen matrix (CO) containing well-defined collagen bundles in developing orthogonal arrays (Bar, 3 μ; ×5500).* **C:** *Control wounds contained fewer fibroblasts than growth-factor–treated wounds and less evidence of collagen synthesis and organization than wounds treated with TGF-β1 (Bar, 3 μ; ×5500).*

the mechanisms leading to enhanced tissue repair in full-thickness excisional open wounds in rabbits were studied by ultrastructural and histochemical techniques. Markedly increased numbers of inflammatory cells, coupled with GAG-containing matrix deposition, were detected in PDGF-BB-treated wounds, compared with controls; in contrast, TGF-β1 appeared to directly accelerate wound collagen synthesis and maturation, perhaps altering the normal course of healing. Myofibroblasts were identified during the active phase of healing (day 10) only in control wounds. Taken together, these findings suggest unique and specific cellular targets and mechanisms of action for PDGF-BB and TGF-β1 in wound repair processes.

Table 2. *Ratio of Inflammatory Cells or Myofibroblasts to Fibroblasts in PDGF-BB- or TGF-β1–Treated Wounds at 10 Days*

	Day 10		Day 21
Wound treatment*	Inflammatory cells/ fibroblasts (%)	Myofibroblasts/ fibroblasts (%)	Myofibroblasts/ fibroblasts (%)
PDGF-BB	35†‡	0	5
TGF-β1	23†	0	2.5
Control	10	12	0

* Three hundred to four hundred cells were counted from 10 to 15 thin sections obtained from two representative wounds per treatment. Control wounds had less total cellularity than growth factor-treated wounds, thus the increased proportion of inflammatory cells in growth-factor–treated wounds indicates an absolute increase of even greater magnitude.

† $P < 0.002$, growth-factor–treated versus control, Chi-square analysis.

‡ $P < 0.01$, PDGF-BB versus TGF-β1.

Wound Cellularity

Platelet-derived growth factor-BB recruited increased numbers of macrophages into the open, granulating wounds, as defined ultrastructurally, consistent with its known potent *in vitro* and *in vivo* chemotactic activity for monocytes/macrophages.[2,24] Macrophages are capable of secreting multiple growth factors at the wound site (eg, PDGF, TGF-β, TGF-α)[25–27] and as shown initially by Liebovich and Ross,[28] are critical for normal tissue repair.[3,4,28] Because PDGF-BB is capable of activating macrophages, including synthesis of TGF-β1 *in vitro* and

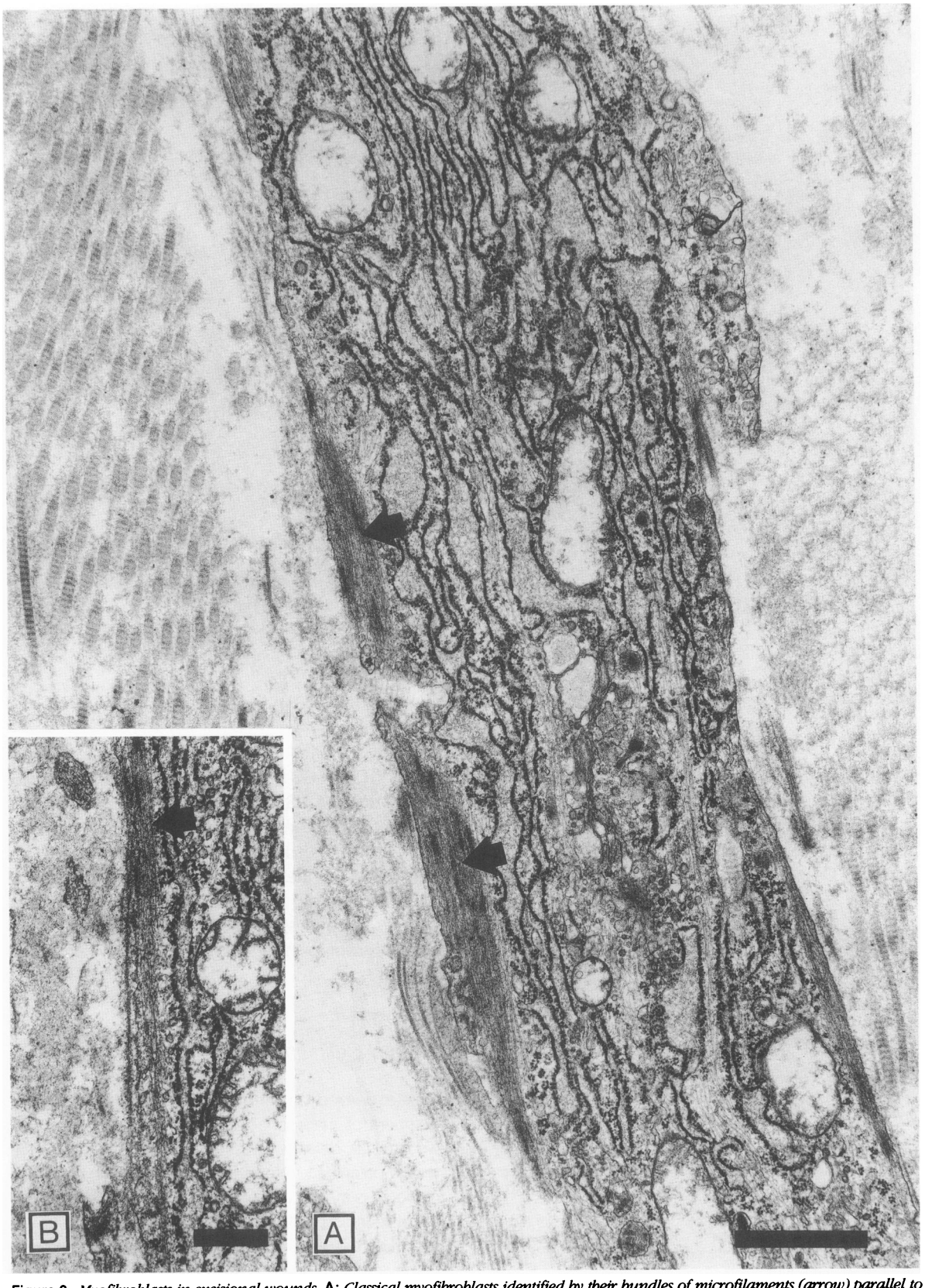

Figure 8. *Myofibroblasts in excisional wounds.* **A**: *Classical myofibroblasts identified by their bundles of microfilaments (arrow) parallel to the longitudinal axis of the cell, were observed in 10-day control wounds (Bar, 1 μ; ×22,000).* **B**: *Myofibroblasts were identified in PDGF-BB–treated 21-day wounds. Fewer myofibroblasts were detected in wounds treated with PDGF-BB or TGF-β1. Additionally, the myofibroblast phenotype (arrow) was significantly less well-defined than in control wounds (Bar, 0.5 μ; ×27,000).*

in vivo,[8,29] PDGF-BB is likely augmenting and amplifying a cascade of growth-factor–mediated activities within the wound milieu through the recruitment and activation of wound macrophages.[3,4] Transforming growth factor-β1, although a potent chemotactic and activating agent for monocytes *in vitro*,[8,30] is less effective than PDGF-BB in recruiting wound macrophages *in vivo* into incisional wounds[8] and excisional wounds (present study). Platelet-derived growth factor-BB thus transiently augments the acute inflammatory phase of tissue repair.

As observed previously using cultured wound explants,[12] PDGF-BB stimulated increased fibroblast proliferation in wounds, as assessed by ultrastructural analysis, in contrast to TGF-β1 and control wounds. Platelet-derived growth factor is the most potent serum mitogen for fibroblasts,[6] in contrast to TGF-β1, which is a bifunctional regulator of fibroblast growth.[7,31] Both growth factors are potent chemotactic agents for fibroblasts,[8,32–34] an activity likely mediating the increased fibroblast influx into normal wounds observed *in vivo*. Of importance, both PDGF and TGF-β activate fibroblasts to synthesize growth factors in addition to extracellular matrix constituents. Platelet-derived growth factor stimulates PDGF-AA and TGF-β1 production[3,8,35]; TGF-β triggers TGF-β and PDGF synthesis.[31,36] Thus through directed cellular recruitment, proliferation, and activation, supraphysiologic doses of PDGF-BB and TFG-β1 initiate cascades of unique activities, including positive autocrine feedback loops, which increase further endogenous growth factor and extracellular matrix synthesis within wounds.

Wound Matrix Deposition

Collagen fibrillogenesis and bundle formation were considerably more prominent at the leading edge of new extracellular matrix deposition in TGF-β1–treated than in PDGF-BB–treated wounds, as assessed by ultrastructural analysis and polarization microscopy (Figures 2, 4). These observations extend those of previous *in vitro* studies that indicate that TGF-β1, but not PDGF, directly stimulates and stabilizes procollagen type I transcription, permitting increased translation.[37–40] The decrease in collagen fibrils present at the leading edge of extracellular matrix in 10-day-old PDGF-BB–treated wounds, in the presence of significantly more extracellular matrix than measured in control wounds, was unexpected and initially puzzling. The PDGF-BB–induced matrix qualitatively resembled control wounds (ie, little collagen at the leading edge of new tissue), however, and contained markedly increased levels of glycosaminoglycans, prominent provisional extracellular matrix constituents present early in wound healing.[41,42] Increased fibronectin may be present as well,[16,43] as PDGF stimulates fibronectin and hyaluronic acid synthesis in fibroblasts *in vitro*.[44–46] Thus the accelerated establishment and augmentation of an enhanced provisional matrix of proteoglycans, hyaluronic acid, and fibronectin in advance of increased collagen accumulation may be a primary mechanism of action of PDGF-BB in early granulating wounds, and is presently under study.

In contrast, TGF-β1–treated wounds appear to deviate from the progressive time-dependent matrix deposition observed in PDGF-BB and control wounds, and stimulate increased levels of collagen, which matures considerably more rapidly. These results are consistent with the finding that TGF-β1 has earlier, but more transient, effects than PDGF-BB in the dermal incisional wound model, where wound breaking strength is a direct measure of net collagen synthesis.[1,3] Preliminary experiments indicate the absence of GAGs and presence of collagen bundles in 5- and 7-day-old TGF-β1–treated excisional wounds, suggesting that TGF-β1 does not simply accelerate tissue repair processes, but actually circumvents provisional matrix deposition (unpublished observations). Further analysis is required to more precisely identify the quantitative and kinetic differences in extracellular matrix synthesis and assembly induced by PDGF-BB or TGF-β1.

Platelet-derived growth factor is a potent stimulator of collagenase expression in fibroblasts.[47] In the present study, PDGF-BB treatment may be stimulating fibroblast collagenase synthesis, perhaps contributing to the observed delayed accumulation of collagen in the presence of increased extracellular matrix, compared with TGF-β1–treated wounds. Increased tissue remodeling early in PDGF-BB–mediated repair may permit an acceleration of ordered net collagen synthesis, leading to the previously observed sustained increased incisional wound breaking strength, in contrast to the transient effect of TGF-β1 treatment.[8] Transforming growth factor β1 decreases collagenase synthesis, stimulates tissue inhibitor of metalloprotinase (TIMP, a collagenase inhibitor),[48,49] and induces fibroblast procollagen type I, fibronectin, and GAG synthesis.[37–40,50,51] Through increased transcription and in some cases mRNA stabilization, TGF-β1 therefore induces new matrix and stabilizes newly formed collagen. Thus, both growth factors accelerate the matrix assembly phase of wound healing, although with different kinetics and clearly independent mechanisms that control net collagen and GAG deposition (Table 3).

Wound Myofibroblasts

Open wounds *in vivo* are only partially healed by new collagen synthesis, ingrowth of neovessels, and cellular proliferation (ie, granulation tissue formation). Another

Table 3. *Summary of the Influence of PDGF-BB or TGF-β1 Treatment Extracellular Matrix Generation in Open Wounds*

Activity	PDGF-BB	TGF-β1	Control
Fibroblast influx*	↑↑	↑	↑
Fibroblast proliferation*	↑↑	↑	↑
Glycosaminoglycan synthesis	↑↑↑	↑	↑
Collagen synthesis	↑↑	↑↑↑	↑
Collagen maturation	↑	↑↑	↑
Myofibroblast generation	↓	↓	↑

* Reference 12.

critical process is wound contraction, in which the edges of a wound are pulled together by forces generated within the wound.[13–15] Thus an open wound on an animal's flank closes largely due to contraction of granulation tissue. Yet contraction after a severe burn can generate a profound deformity, and scar contraction in cirrhosis may be fatal. Therefore, either stimulation or inhibition of wound contraction may be desirable biologic processes.

The role of myofibroblasts in contracting wounds, and the influence of growth factors on this process, were not evaluable in the present experiments, as this is a noncontracting wound model. The possible inhibition of myofibroblasts, however, the likely motive force for wound contraction,[13–15] in growth-factor–treated wounds is an intriguing finding with potentially important biologic ramifications. This cell is defined and identified by its electron microscopic appearance, sharing characteristics of fibroblasts and smooth muscle cells, and containing bundles of 6- to 8-nm microfilaments with electron-dense bodies.[13–15,52] *Ex vivo* pharmacologic testing has shown that granulation tissue containing myofibroblasts is contracted or relaxed in response to agonists and antagonists of smooth muscle contraction, respectively.[13] More recently, however, monoclonal antibodies to fibroblast, but not smooth muscle isoforms of F-actin, have demonstrated significant staining of the cytoplasm of myofibroblasts in wound granulation tissue, suggesting their derivation from wound fibroblasts.[16,18–20] Significantly, electron microscopic quantification of the myofibroblast population has demonstrated that it peaks during the active phase of experimental wound contraction in pigs and rodents.[13,14,16,50] The present data, which demonstrate myofibroblasts during the active phase of healing in control wounds (day 10), suggest an inverse relationship between growth-factor–induced fibroblast secretory activities and myofibroblast formation (Table 3). This hypothesis may be tested best in a contracting wound model.[53]

In support of this hypothesis, PDGF-BB was recently shown ineffective in stimulating normal wound contraction.[53,54] Clark and coworkers[55] recently found PDGF-BB stimulated contraction of fibroblast-containing collagen matrices. This assay is considered the possible *in vitro* correlate of wound contraction, although this relationship has not been established. The present results suggest that the stimulatory effects on matrix deposition by growth factors may supersede their influence on contraction *in vivo*. It is unlikely that growth factors are simply delaying myofibroblast differentiation, although this possibility cannot be ruled out by the present experiments, as a small number of myofibroblasts were found in growth-factor–treated wounds at day 21 (Figure 7). However, by day 21, the wound has fully healed; thus the peak period of myofibroblast activity probably would be concluded. Peak numbers of myofibroblasts would be expected at day 10, when no myofibroblasts were observed in growth-factor–treated wounds, but were detected in control wounds, as the wounds have not yet closed.

Transforming growth factor β1 is a known potent inhibitor of cell differentiation, including myoblast differentiation.[56,57] Although evidence points to the fibroblast derivation of the myofibroblast,[19,20] it is intriguing to speculate that TGF-β1 may inhibit transcription of potential contractile proteins required for the induction of the myofibroblast phenotype.

In summary, single supraphysiologic doses of PDGF-BB and TGF-β1 have profound, distinct influences on the generation of extracellular matrix and decrease myofibroblast formation in experimental excisional granulating wounds. Platelet-derived growth factor-BB markedly augments the normal acute inflammatory phase of wound healing, which amplifies a cascade of increased endogenous growth factor and provisional matrix synthesis by macrophages and fibroblasts. In contrast, TGF-β1 may alter the normal course of healing and has a more direct effect on the collagen synthesis phase of tissue repair, extending previous observations made in the incisional wound model. The apparent inhibition of myofibroblast formation in the excisional model suggests certain useful biologic correlates. Platelet-derived growth factor-BB and TGF-β1 may be useful for stimulation of new extracellular matrix in wounds in which contraction should be minimized. Thus growth factors that regenerate missing tissue may obviate the need for contraction, thus augmenting the ultimate structural integrity of the healed wound.

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